Isolation and Physiochemical Properties of Protein-Rich Nematode Mitochondrial Ribosomes†

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Received October 8, 2004; Revised Manuscript Received May 6, 2005

ABSTRACT: In the present study, mitochondrial ribosomes of the nematode *Ascaris suum* were isolated and their physiochemical properties were compared to ribosomes of *Escherichia coli*. The sedimentation coefficient and buoyant density of *A. suum* mitochondrial ribosomes were determined. The sedimentation coefficient of the intact monosome was about 55 S. The buoyant density of formaldehyde-fixed ribosomes in cesium chloride was 1.40 g/cm^3, which suggests that the nematode mitoribosomes have a much higher protein composition than other mitoribosomes. The diffusion coefficients obtained from dynamic light scattering measurements were $(1.48 \pm 0.04) \times 10^{-7}$ cm^2 s^{-1} for 55 S mitoribosomes and $(1.74 \pm 0.04) \times 10^{-7}$ cm^2 s^{-1} for the 70 S *E. coli* monosome. The diameter of mitoribosomes was measured by dynamic light-scattering analysis and electron microscopy. Though the nematode mitoribosome has a larger size than the bacterial ribosome, it does not differ significantly in size from mammalian mitoribosomes.

Ribosomes are ubiquitous ribonucleoprotein particles that translate genetic messages into proteins. The sedimentation coefficients of the intact monosome, large subunit, and small subunit of a bacterial ribosome (*Escherichia coli*) are 70, 50, and 30 S, respectively (*1*). Recently, considerable research has focused on understanding the structure and function of mammalian mitochondrial ribosomes (mitoribosomes[1]). The mammalian mitoribosome is a highly protein-rich particle with a small sedimentation coefficient of about 55 S, which consists of one large (39 S) and one small (28 S) subunit (*2*−*4*). These subunits contain 16 and 12 S rRNAs, respectively, but no bacterial 5 S rRNA counterparts. The total length of mitochondrial rRNA is about a half that of bacterial rRNA (*5*), but the protein content of mammalian mitoribosome is 3-fold higher than that of the bacterial ribosome (*6*, *7*). Almost a half of the rRNA contained in the bacterial ribosome is replaced with proteins in the mammalian mitoribosome.

Previous studies have shown that the nematode mitoribosome is thought to contain rRNA of highly reduced size compared to the mammalian and bacterial ribosomes (*8*, *9*). The combined sizes of rRNA genes from both the large and small subunits are 4446, 2536, and 1677 bases for bacteria (*Escherichia coli*), mammalian mitochondria (*Mus musculus*), and nematode mitochondria (*Ascaris suum*), respectively (*9*−*12*). This may imply that some of the nematode mitochondrial ribosomal proteins are recruited to compensate for the reduced size of the mitochondrial rRNAs (*13*). To date, analyses of animal mitoribosomes have been primarily limited to mammalian species, bovine and rat liver, and so forth. Extensive analysis of mammalian mitoribosome revealed its function (*14*−*16*) and structure (*17*). Some noteworthy differences have been identified in nematode mitoribosomes: (1) the rRNA component is among the smallest characterized thus far (*8*, *9*, *18*) and (2) nematode mitoribosomes interact with unusual tRNAs lacking the T- or D-arm (*8*, *19*−*23*), and there exist two unusual EF-Tu's compensating for the structural deficiencies of those tRNAs (*24*, *25*). However, no studies have yet reported characterization of intact nematode mitoribosomes due to the difficulty in their preparation. In the present study, we prepared mitoribosomes from *A. suum*, a nematode which is a pig intestinal parasite, and have characterized the physiochemical properties to gain insights into nematode mitoribosome structure and function.

MATERIALS AND METHODS

Materials and Chemicals. A. suum adult worms were purchased from a local slaughter house (Tokyo, Japan). The worms were frozen at −80 °C or dissected to prepare the body wall muscle with cuticle attached and then stored at −80 °C. When the muscle was prepared, care was taken to remove the entire enteron intact, since it contains many

† This work was supported by Grants-in-Aids from the Ministry of Culture, Sports, Science and Technology, Japan to T.O., K.K., Y.-i.W., and K.W.

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[1] Abbreviation: mitoribosome, mitochondrial ribosome.

10.1021/bi047833c CCC: $30.25 © 2005 American Chemical Society
Published on Web 05/27/2005

Table 1: DNA Sequences of Hybridization Probes for Mitoribosomes

rRNA	probe sequence (from 5′ to 3′)
A. suum mitochondrial rRNA (large subunit)	CTACCTTAATGTCCTCACGCTAAG
	ACTGCCAGTAAAAAAAGTTACAAA
	ACATTCTACCCTAACCTAAAATCC
	TCTCCTCCCTTTAAGAACTCCAAC
	AAATTTCCTTTCGTACTTTCAGTA
A.suum mitochondrial rRNA (small subunit)	TTCCCGATTTCACCCTAAAAAACA
	AGATATTAATCAGGTACTAATCTGA
	TCATTCACCACTTACAGTTTAAA
	TACAACTTTACTCCTGCGTAGTTA
	ATGAGGGTTCTCAATTACTACTC
bovine mitochondrial rRNA	ATTTATCCAAGCACACTTTCTGTCA
	CTGGGCAGGCAATGCCTCCAATAC
	TGATCCAACATCGAGGTCGTAAAC
	CCTATT

ribonucleases and proteases. In some cases, the muscle was prepared from living worms after maintenance of the worms in Ringer's solution (0.85% (w/v) NaCl, 0.024% (w/v) KCl, 0.016% (w/v) $CaCl_2 \cdot 2H_2O$, and 0.01% (w/v) $NaHCO_3$) overnight at room temperature, and the tissue was used without freezing−thawing. Ultrapure sucrose was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals were of analytical grade.

Sequences for DNA probes (four large subunit probes and four small subunit probes) to detect *A. suum* rRNA are shown in Table 1. A mixture of the four large subunit probes or four small subunit probes at a total concentration of 2 pmol/μL was used in the hybridization experiments to detect the mitoribosomes. Sequences of probes for bovine mitochondrial rRNA are also shown (Table 1).

Preparation of A. suum Mitochondria. Preparation of *A. suum* muscle mitochondria was based on the procedure of Ernster and Nordenbrand (*26*), with a modification by Yamashita et al. (*27*) to obtain higher yields, as determined by the specific activity of succinate dehydrogenase, a marker enzyme of the mitochondrial inner membrane. The muscle, prepared as described above, was minced with scissors and transferred to a Waring blender (Cell Master CM-100, Iuchi, Japan) with equal volumes of Chappell Perry buffer (0.1 M KCl, 50 mM Tris-HCl, 1 mM ATP, 5 mM $MgSO_4$, and 1 mM EDTA, pH 7.4 (*28*)). After running the blender slowly for 1 min and at the maximum speed for an additional minute, followed by homogenization with a Hiscotron (Kinematica AG, Lucerne, Switzerland) for 2 min at the maximum speed, the muscle was finally homogenized using a 50-mL loose glass-Teflon homogenizer. Cells, cell debris, and nuclei were removed by centrifugation of the homogenate at 750*g* for 10 min. Mitochondria were then recovered from the supernatant by centrifugation at 17 000*g* for 15 min. For digitonin treatment to disrupt the outer membranes of mitochondria, the mitochondrial pellets were suspended and homogenized in 50 mL of IM buffer (0.07 M sucrose, 2 mM Hepes-KOH, 1 mM EDTA, 0.22 M mannitol, pH 7.6, and 0.2 mg/mL digitonin) and stirred for 15 min. The suspension was then centrifuged at 20 000*g* for 10 min to recover the digitonin-treated mitochondria.

Preparation of Mitochondrial and E. coli Ribosomes. The digitonin-treated mitochondria were homogenized in 40 mL of RE buffer (100 mM KCl, 15 mM Tris-HCl, 0.8 mM EDTA, 15 mM $MgCl_2$, 1.6% Triton X-100, 0.26 M sucrose, 50 μM spermine, 50 μM spermidine, 6 mM 2-mercaptoethanol, and 100 μM phenylmethylsulfonyl fluoride (PMSF), pH 7.8) and stirred at 4 °C for 15 min. After centrifugation at 21 000 rpm for 20 min in a Beckman 70Ti rotor (Beckman, San Diego, CA), the supernatant (S30) was collected and overlaid onto a 10 mL sucrose cushion (100 mM KCl, 20 mM $MgCl_2$, 20 mM triethanolamine-HCl, 1 M sucrose, 1% Triton X-100, and 6 mM 2-mercaptoethanol, pH 7.5) and centrifuged at 38 700 rpm for 18 h in a 70Ti rotor. The yellowish pellet (crude ribosomes) was collected and suspended in a minimal volume of Basic Buffer (20 mM Tris-HCl, 20 mM $MgCl_2$, 80 mM KCl, and 6 mM 2-mercaptoethanol, pH 7.8) for storage at −80 °C. We prepared the ribosome using 100−300 g of *A. suum* muscle, and the average yield for crude mitoribosome was 6 A_{260} units from 100 g of *A. suum* muscle.

E. coli ribosomes were prepared by the methods described previously (*29*) and used in reference experiments.

Purification of Ribosomes by Sucrose Density Gradient Centrifugation. A 6−38% linear gradient of sucrose in Basic Buffer was used to fractionate mitoribosomes. As a reference, a 6−38% linear gradient of sucrose in buffer A (20 mM Hepes-KOH, 1 mM $Mg(OAc)_2$, 200 mM NH_4Cl, and 6 mM 2-mercaptoethanol, pH 7.6) was used for *E. coli* ribosome fractionation. About 600 μL of crude ribosomes (5−20 A_{260} units) was applied to the sucrose gradient and centrifuged in a Beckman SW28 rotor at 20 000 rpm for 16 h at 4 °C. Fractions of 500 μL were collected from the bottom of the tube and absorbance at 260 nm (*E. coli*) or dot hybridization radioactivity (*A. suum*) was measured. The purified peak fractions from the sucrose density gradient centrifugation were dialyzed overnight against three changes of Basic Buffer at 4 °C and concentrated in an Amicon ultracentrifugal filter device (Millipore Corporation, Billerica, MA) with a 30 kDa nominal molecular weight limit prior to storage.

Detection of Mitochondrial Ribosomes by Dot Blot Hybridization. Two microliters of each fraction was applied to a Nylon Hybond-N^+ membrane (Amersham Biosciences, Piscataway, NJ). After drying the membrane, the sample was fixed to the membrane by UV cross-linking twice with energy of 120 000-μJ/cm^2. The membrane was incubated in prehybridization buffer (0.9 M NaCl, 0.09 M sodium citrate, 0.1% SDS, 0.1% Ficoll, 0.05% poly(vinylpyrrolidone), and 0.1% acetylated BSA) for 2 h at 42 °C. The membrane was soaked in 10 mL of hybridization buffer (0.9 M NaCl, 90 mM Tris-HCl, 6 mM EDTA, and 0.3% SDS, pH 8.0) containing 10 μL of radioactively labeled probe. Probes were labeled by incubating 0.5 pmol DNA with one unit of T4 polynucleotide kinase and 10 μCi of γ-[^{32}P]ATP at 37 °C for 1 h. The membrane was incubated with the probes for 16 h at 50 °C and then washed with SSC solution (0.15 M NaCl and 0.015 M sodium citrate) three times for 15 min. The membrane was removed, dried, and exposed to X-ray film to detect the radioactive spots. The intensity of the spots on the film was calculated using Image Gauge Software (FUJIFILM, Tokyo, Japan).

Dynamic Light Scattering Analysis. The purified 70 S peak fraction for *E. coli* ribosomes and the 55 S fraction for *A. suum* mitoribosomes, obtained by sucrose density gradient centrifugation, were used for dynamic light scattering experiments by Dynamics V5 (Protein Solutions, Charlottesville,

VA). The intensity-normalized photocount autocorrelation function was used to determine the translational diffusion coefficient D_{20} by cumulants analysis (*30*−*32*). The D_{20} was corrected for solvent viscosity to water (i.e., $D_{20,w}$). The $D_{20,w}$ was subsequently employed to determine the particle size (Stokes radius) utilizing the Einstein−Sutherland equation (*33*).

Electron Microscopy of Mitoribosomes and E. coli Ribosomes. The 55 S mitoribosomes and 70 S *E. coli* ribosomes obtained from the peak maxima of sucrose gradients were serially diluted with water and adsorbed onto carbon-coated grids. The grids were again washed with deionized water and negatively stained with 0.5% (w/v) uranyl acetate (*34*, *35*). Micrographs were taken at a magnification of 200 000 with a Hitachi H-7600 transmission electron microscope (Hitachi, Tokyo, Japan) operated at 100 keV. Particle diameter measurements were randomly recorded from digitized images of about 50 ribosomes using Image Tool software Version 3 (University of Texas Health Science Center, Houston, TX). The length and width of particles were also assessed when ribosomes were orientated in the frontal view. Results are expressed as the mean ± SD.

Buoyant Density Analysis in Cesium Chloride. The buoyant density analysis is based on the methods of Huxley et al. (*36*) and Spirin et al. (*37*) The purified ribosomes (0.02 A_{260} unit) were carefully suspended in 0.01 M potassium phosphate buffer (pH 7.2) containing 0.01 M $MgCl_2$, and the suspension was mixed with an equal volume of 4% neutralized formaldehyde solution prepared in the same buffer. The mixture was kept on ice for 45 min to ensure complete fixation; subsequently, 2 mL of the mixture was added to 9 mL of CsCl solution and centrifuged in a Beckman SW41 Ti rotor (Beckman Instruments, San Diego, CA) at 33 000 rpm for 48 h at 4 °C. Fractions were collected from the bottom of the tube to measure the refractive index. RNA was extracted from fractions with ISOGEN (NIPPON GENE, Toyama, Japan) and chloroform and used for dot blot hybridization measurements to detect the ribosomes.

Buoyant density was determined using the equation $d = 10.9276n - 13.593$, where d is the density and n is the refractive index of the fraction at 20 °C (*38*). In this measurement, bovine mitoribosomes were used as reference sample to validate the techniques. A mixture of three DNA probes described above was used to detect the bovine mitoribosomes.

RESULTS AND DISCUSSION

Isolation and Purification of Nematode Mitoribosomes. A. suum mitoribosomes were prepared as described in the experimental procedures and their sedimentation coefficients were determined by comparing their speed of sedimentation to those of *E. coli* ribosomes on sucrose density gradients. *E. coli* ribosomal fractions were detected by absorbance at 260 nm. As shown in Figure 1, the *E. coli* monosome (70 S) and large subunit (50 S) peaks were observed clearly. A small subunit peak of 30 S was also detected, although the peak was not distinct. Fractions collected from *A. suum* were subjected to dot hybridization with DNA probes specific to the large subunit or small subunit rRNA of mitoribosomes, and radioactivity of each dot was measured. There were two main peaks (72 and 55 S) and one minor peak with a small

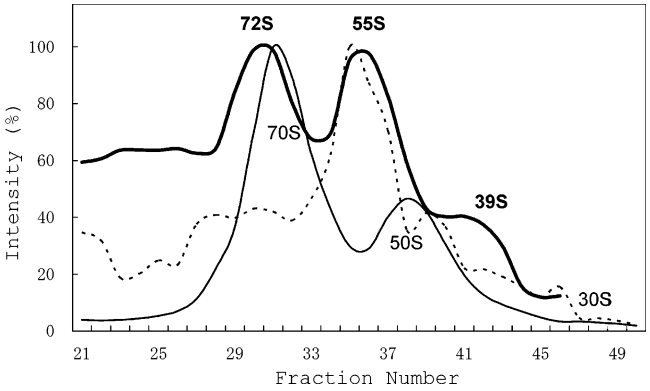

FIGURE 1: Sucrose density gradient analysis of mitochondrial and *E. coli* ribosomes. Crude ribosome samples were applied to a 6−38% sucrose density gradient and centrifuged in the Beckman SW28 rotor at 20 000 rpm for 16 h at 4 °C. Fractions were collected from the bottom of the tube and their A_{260} absorbance (*E. coli*, solid line) and dot hybridization radioactivity using large subunit probes (*A. suum*, broad solid line) and small subunit probes (*A. suum*, dashed line) were measured. Intensities of A_{260} absorbance and dot hybridization radioactivity were normalized by the highest intensity (as 100%). Numbers indicate the sedimentation coefficients for *E. coli* and calculated values for mitoribosomes, respectively.

sedimentation coefficient (39 S) (Figure 1). By using the fraction number and the sedimentation coefficients of *E. coli*, we roughly calculated the sedimentation coefficients of the *A. suum* mitoribosome peaks to be 72, 55, and 39 S. The 55 S peak fraction was detected by both large subunit probes and by small subunit probes (Figure 1) and represents the monosome. The 39 S peak fraction contains the large subunit, because the 39 S fraction was detected with the large subunit probes (Figure 1) but not by the small subunit probes (Figure 1). The 72 S peak fraction was mainly detected by the large subunit probes (Figure 1). The 72 S particles had uniform spherical shape (about 35 nm × 40 nm) when they were observed by electron microscopy. Although we could not describe what the 72 S particles represent, the particles probably are not monosomes because they have too large a size and too large a sedimentation coefficient (in mammalian cases, sedimentation coefficient of mitoribosomes are 55 S (*2*−*4*), and size of rat mitoribosome is about 24 nm × 26 nm (*7*)). Since the 72 S particles have uniform shape, they do not seem to be aggregated mitoribosomes. Therefore, we speculated that 55 S peak represents monosome, composed of one-to-one complex of large and small subunits, and 72 S has some distinctive composition from 55 S.

The purified peak fractions from the sucrose density gradient centrifugation were dialyzed overnight against three changes of the Basic Buffer to ensure the removal of sucrose and then used for other experiments. From about 6 A_{260} units of crude mitoribosome prepared as described in Materials and Methods from 100 g of *A. suum* muscle, about 0.04 A_{260} unit of 55 S mitoribosome fraction was obtained. Northern hybridization analysis using agarose gel and DNA probes for the rRNA detection suggested that each of the purified peak fractions included the large subunit rRNA of an appropriate size, although it degraded easily during RNA extraction (data not shown).

Determination of Diffusion Coefficients by Dynamic Light Scattering. Dynamic light scattering experiments were carried out with the sucrose density gradient purified 55 S mitoribosomes. To validate the techniques employed to characterize

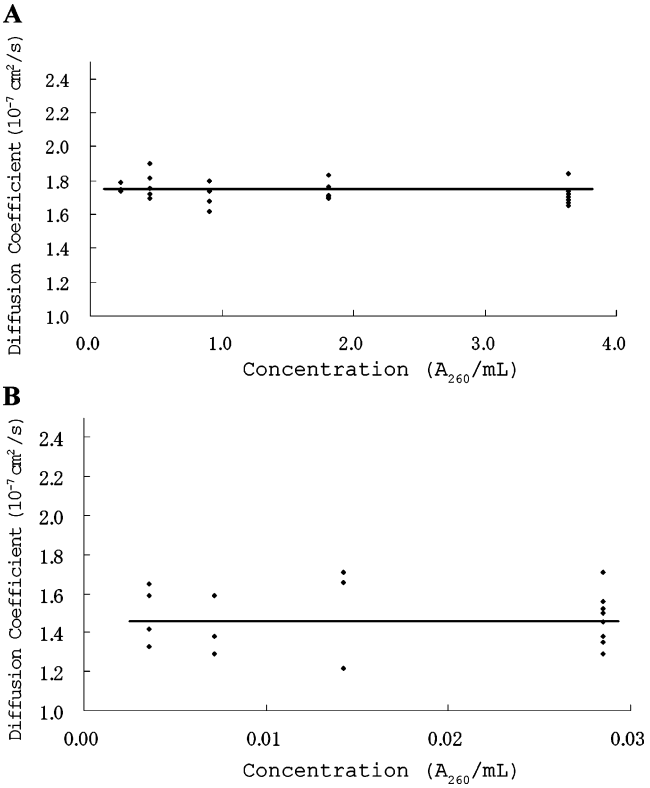

FIGURE 2: Diffusion coefficient measurements of mitochondrial and *E. coli* ribosomes. Dynamic light scattering analysis was carried out on ribosomes obtained from the peak fractions of sucrose density gradients. The straight line between each set of data points indicates the mean diffusion coefficient. (A) *E. coli* 70 S ribosomes, (B) *A. suum* mitoribosomes.

the mitoribosomes, we applied the standard procedures to the characterization of the physiochemical properties of the well-described 70 S *E. coli* ribosomes (*39*, *40*). Experiments were performed independently three times and similar results were obtained. As shown in Figure 2, the translational diffusion coefficients obtained from dynamic light scattering experiments were $(1.742 \pm 0.037) \times 10^{-7}$ cm^2 s^{-1} for the 70 S *E. coli* monosome (Figure 2A) and $(1.475 \pm 0.038) \times 10^{-7}$ cm^2 s^{-1} for *A. suum* mitoribosomes (Figure 2B). The $D_{20,w}$ was independent of concentration over the range of 0.0036−0.0285 A_{260} unit/mL for mitoribosomes and 0.227−3.630 A_{260} unit/mL for *E. coli* ribosomes and therefore behaved as an ideal noninteracting system within the above concentration ranges. The ideal concentration range for the mitoribosomes is much lower than that of *E. coli* ribosomes, because the mitoribosomes showed much weaker stability and aggregated easily in comparison with *E. coli* ribosomes.

The diameters obtained from calculating the Stokes radius were 30.8 ± 0.7 nm for nematode mitoribosomes and 25.4 ± 0.4 nm for *E. coli* ribosomes. In both cases, especially in the mitoribosome case, minor peaks with a diameter greater than 100 nm, possibly implying aggregation of ribosomes, were present (less than 1% in *E. coli* and about 5−10% in mitoribosomes).

Electron Microscopy of Mitoribosomes and E. coli Ribosomes. Figure 3 shows the electron micrographs of negatively stained mitoribosomes (left) and *E. coli* (right) ribosomes. The dimensions of the mitoribosomes were (26.4 ± 0.9) nm × (22.9 ± 0.9) nm, resulting in an axial ratio of 1.15. The *E. coli* ribosomes showed dimensions of (21.7 ± 0.4) nm × (19.5 ± 0.8) nm, resulting in an axial ratio of 1.11. These values for the *E. coli* ribosome are very similar to those previously described in the literature (*7*, *40*).

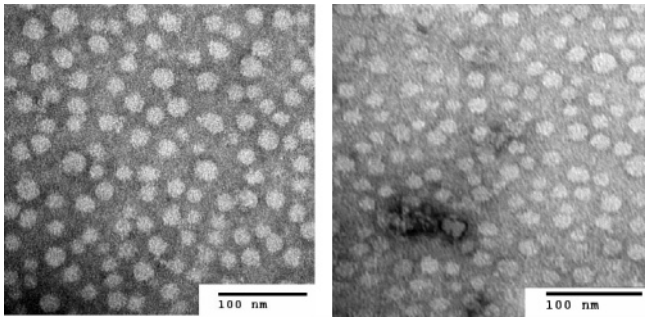

FIGURE 3: Electron micrographs of 55 S mitochondrial (left) and 70 S *E. coli* ribosomes (right). Electron microscopy was carried out on *A. suum* mitoribosomes and *E. coli* ribosomes obtained directly from the peak fractions of sucrose density gradients. Ribosomes were negatively stained with uranyl acetate and visualized at a magnification of 200 000.

Dynamic light scattering experiments also provided an estimate of ribosome diameter. We found that the diameters obtained from dynamic light scattering experiments were larger than that from electron microscopy, but the differences in both *E. coli* ribosomes and the nematode mitoribosomes were comparable (about 17% in both cases). This may be due to the contamination of the aggregates with diameter peak greater than 100 nm.

Determination of Buoyant Density and Calculation of Mitoribosome Composition. As described in the experimental procedure, the purified ribosomes were fixed with neutralized formaldehyde solution. Formaldehyde treatment has been employed by many authors in stabilization of ribosomes in concentrated salt solutions [first reported by Spirin et al.-(*37*)], and it has been demonstrated that the fixation does not alter the sedimentation properties of the particles (*37*). After centrifugation, fractions were collected for radioactive and refractive index measurements. First, we measured the refractive index of fractions collected from bovine mitoribosomes as a standard reference. The buoyant density of bovine mitoribosomes was 1.41 g/cm^3 (Figure 4B), the same as previously reported data (*6*, *7*), thus, validating our experimental technique. By using the same method, we determined the buoyant density of nematode mitoribosomes to be 1.40 g/cm^3 (Figure 4A).

The composition of RNA and protein contained in nematode mitoribosomes can be calculated according to the following equation (*41*, *42*):

$$\bar{\nu} = 1/d = \frac{\%\text{RNA}}{100}(\bar{\nu}_{\text{RNA}}) + \frac{\%\text{Protein}}{100}(\bar{\nu}_{\text{Protein}})$$

In the above equation, $\bar{\nu}$ is the partial specific volume of the ribosome, and can be estimated as the inverse of the buoyant density, where $\bar{\nu}_{\text{RNA}}$ is 0.53 mL/g (*42*, *43*), $\bar{\nu}_{\text{Protein}}$ is 0.74 mL/g (*42*, *44*), and d is the buoyant density of the ribosome (1.40 g/mL). By using this calculation, we determined the compositions of RNA and protein in nematode mitoribosomes to be 12% and 88%, respectively. The proportions of RNA and protein in the mammalian mitoribosomes were calculated to be 25% and 75%, respectively, based on the reported mass of mitoribosomal proteins (*45*) and the sum of mitoribosomal 12 and 16 S rRNA species

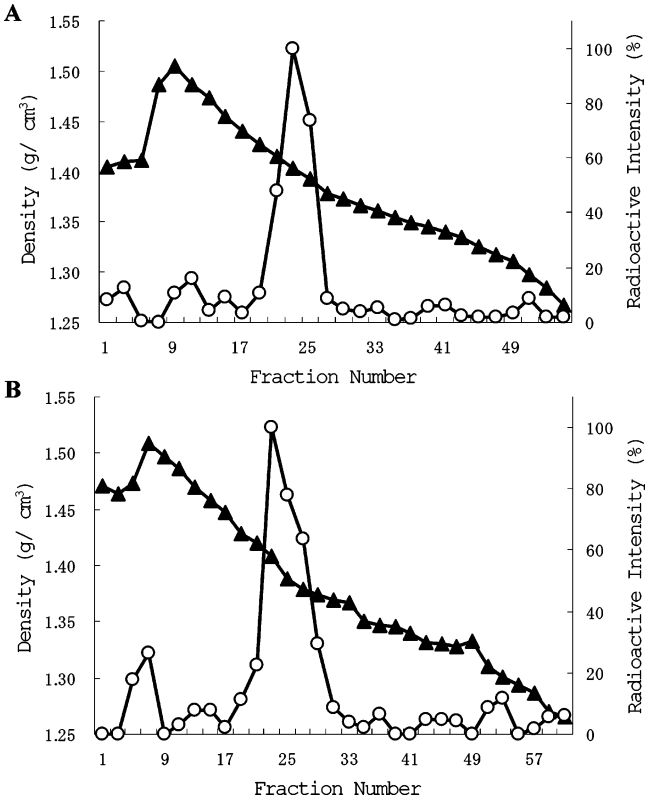

FIGURE 4: Determination of the buoyant density of nematode mitoribosomes. Purified ribosomes were fixed with formaldehyde and centrifuged in a cesium chloride gradient (1.26−1.50 g/cm^3) to determine the buoyant density. To validate the techniques employed to characterize the nematode *A. suum* mitoribosomes (A), bovine mitoribosomes were used as a standard (B). Closed triangles show buoyant density. Open circles show radioactivity by dot blot hybridization.

(*46*). But, by using the reported data of buoyant density (1.41 g/mL, (*7*)), the mammalian mitoribosome composition would be calculated to be 15% and 85%, respectively. These results reveal a tendency toward a lower value for the proportion of RNA in the ribosome when calculated from the buoyant density.

In the present study, we purified mitoribosomes from the parasitic nematode *A. suum* and studied their physiochemical properties compared to those of *E. coli* ribosomes. The sedimentation coefficient of nematode mitoribosomes was determined to be approximately 55 S, which was almost the same as that of the mammalian mitoribosomes. By dynamic light scattering and electron microscopy experiments, the diameter of nematode mitoribosomes was also determined. The nematode mitoribosome has a larger size than the bacterial ribosome but is not much different from that of mammalian mitoribosomes, which were observed by electron microscopy to be (26.2 ± 0.4) nm × (23.6 ± 0.4) nm (*7*). The composition of RNA in nematode mitoribosomes was determined to be 12% in our study, although the value estimated from the buoyant density tends to be lower than values obtained from other measures. The fraction of RNA in bacterial and rat liver mitochondrial ribosomes has previously been determined to be 62% (*37*) and 25% (*7*), respectively. Previous reports have also shown that the nematode mitoribosome contains rRNA of highly reduced size compared to the mammalian counterparts (*9*). Our studies indicate that nematode mitoribosomes have lower RNA and higher protein composition than data reported for mammalian counterparts. This may indicate that some of the nematode mitochondrial ribosomal proteins have been recruited to compensate for the reduced size of the mitochondrial rRNA. In future studies, it will be especially important to identify mitoribosomal proteins and associated proteins to understand the structure−function relationships that have evolved in this unusually protein-rich mitoribosome.

ACKNOWLEDGMENT

We thank Drs. T. Suzuki and M. Terasaki for providing us with bovine mitoribosomes, Dr. Aiko Hirata for technical help with the electron microscopy experiments, and members of Department of Biomedical Chemistry, the University of Tokyo, especially Drs. A. Osanai, T. Yamashita, Mr. S. Ohara, and Ms. N. Shinjyo, for their help in preparing *A. suum* muscle and mitochondria, and Drs. K. Yamamoto and J. Ando (Department of System Physiology, Graduate School of Medicine, University of Tokyo) for use of their facility.

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BI047833C